REMARKS

Claims 1, 2, 4, 6, 7, 10-16, 18, 20-24 and 43-49 are pending. No new matter has been added by way of the present amendments. For instance, claims 1 and 15 have been amended to clarify the language relating to the nature of the amino acid substituted at position 13. These amendments necessitated parallel amendments to claims 7, 20 and 21. Claim 22 was amended to clarify the nature of the nucleotide replacement at position 37. This amendment necessitated a parallel type of amendment to claim 23. New claim 48 is supported by pending claim 1 with the difference being the particular wash conditions supported by the present specification at page 46, lines 8-15, in particular the wash conditions are taken from p. 1.103 of "Molecular Cloning 2nd Edition" (Cold Spring Harbor Laboratory Press, 1989), attached as Reference 2. New claim 49 is the same as new claim 48, with the exception that the nucleotide sequence subject to the hybridization is defined in terms of SEQ ID NO:4 rather than a sequence encoding SEQ ID NO:1. Accordingly, no new matter has been added.

In view of the following remarks, Applicants respectfully request that the Examiner withdraw all rejections and allow the currently pending claims.

Issues under 35 U.S.C.§ 112, second paragraph

The Examiner has rejected claims 1, 2, 4, 6, 7, 10-16, 18, 20-24 and 43-47 under 35 U.S.C. § 112, second paragraph for the reasons recited at pages 2-3 of the outstanding Office Action. Applicants respectfully traverse each of these rejections.

First, the Examiner has rejected claims 1, 7 and 15 for reciting "corresponding to." The Examiner asserts that it is unclear whether the corresponding amino acid, in the part of the protein encoded by the DNA fragment, is valine before it is substituted with another amino acid. Applicants traverse and submit that the claims have been amended to recite that the sequence "encodes an amino acid sequence in which the amino acid at the position corresponding position 13 of SEQ ID NO:1 is an amino acid other than valine." Therefore, the nature of the amino acid prior to the substitution is irrelevant; rather the claims recite the current nature of the amino acid in position 13. Thus, this rejection is moot. Reconsideration and withdrawal thereof are respectfully requested.

Second, the Examiner has rejected claim 1 asserting that there is insufficient antecedent basis for the recitation of "the protein" in line 10 of item (2). Applicants traverse and submit that the recitation of "the protein" no longer appears

in the amended version of the claims. Thus, this rejection is moot. Reconsideration and withdrawal thereof are respectfully requested.

Third, the Examiner asserts that the recitation of "encodes the part of the protein in which the amino acid corresponding to valine at position 13 of SEQ ID NO:1, which is substituted by another amino acid" is confusing. The Examiner has also rejected similar language in claim 15. Applicants traverse and submit that, as indicated above, this phrase in claim 1 has been amended to clarify the nature of the substitution. A similar amendment has been made to claim 15. Thus, these rejections are moot. Reconsideration and withdrawal thereof are respectfully requested.

In view of the above, Applicants respectfully submit that the present claims fully satisfy the requirements of 35 U.S.C. § 112, second paragraph. The Examiner is therefore respectfully requested to withdraw these rejections.

Issues under 35 U.S.C. § 112, first paragraph

Written Description

The Examiner has rejected claims 1, 2, 4, 6, 7, 10-16, 20-24 and 43-47 under 35 U.S.C. § 112, first paragraph for the reasons recited at pages 4-5 of the outstanding Office Action. Applicants respectfully traverse this rejection.

The Examiner maintains that "a part of the protein" does not confer PPO resistance. For instance, the Examiner asserts that DNA fragments as small as 2.6 kb would not encode an entire PPO protein. Applicants submit that it is unnecessary to encode the entire protein. In fact, the Xho/PmaC2.6 fragment (approximately 2.6 kb) contained only a portion of the mutated PPO gene that is resistant to PPO-inhibiting herbicides, but the fragment, when "introduced" into a herbicide-sensitive cell, was able to confer PPO resistance (Example 7, specifically page 50, lines 31 to 37 of the specification). The fragment is integrated by homologous recombination into the herbicide-sensitive PPO gene. The herbicide-sensitive PPO gene in the recipient cell is then converted into a mutant PPO gene, and expressed a mutant PPO that is resistant to PPO-inhibiting herbicides.

The Examiner also maintains that the hybridization and wash conditions in the claims are insufficient. Applicants disagree and submit that the hybridization recited in claims 1 and 15 is carried out by using a partially homologous probe. Partially homologous probes are used to detect DNA clones that are related, but not identical, to the probe sequences (pp. 8.47, the 2nd paragraph of attached Reference 1, which is taken from "Molecular Cloning 2nd Edition" (Cold Spring Harbor Laboratory Press, 1989). The attached Reference 1 (pp.8.47,

the 2nd paragraph) teaches that, hybridization with partially homologous probe in solvents containing formamide, is followed by wash at 50°C in 6XSSC, 0.5% SDS. Comparing with this wash condition, the wash condition recited in the claims 1 and 15 uses lower salt concentration, which means that it is a more stringent salt concentrate condition than those taught in the above Reference 1.

Applicants further point out that Fourgoux-Nicol et al., cited by the Examiner, does not show that the wash temperatures that do not correspond to high stringency conditions allow the hybridization of unrelated sequences. Thus the conditions are sufficient.

Accordingly, those of skill in the art fully understand that, at the time of filing, Applicants were in possession of the invention currently claimed. Reconsideration and withdrawal of this rejection are respectfully solicited.

Enablement

The Examiner has rejected claims 1, 2, 4, 6, 7, 10-16, 20-24 and 43-47 under 35 U.S.C. § 112, first paragraph for the reasons recited at pages 5-7 of the outstanding Office Action. Applicants respectfully traverse this rejection.

As discussed above, the "fragments" (or "part of the protein") recited in the claims are capable of conferring PPO

resistance when introduced into a herbicide-sensitive cell. Further, the hybridization conditions are sufficiently stringent.

Concerning the state of homologous recombination, as disclosed in the specification (page 17, lines 6-11), plant cells resistant to PPO-inhibiting herbicides, due to the presence of the altered PPO coding sequence, may be isolated by growing the population of the plant cells on media containing an amount of PPO-inhibiting herbicide which normally inhibits growth of the untransformed plant cells.

Accordingly, one of ordinary skill in the art may screen plant cells resistant to PPO-inhibiting herbicides efficiently, even if the frequency of homologous recombination in the plant cells is low. Accordingly, those of skill in the art are fully able to make and use the presently claimed subject matter without undue experimentation. Reconsideration and withdrawal of this rejection are requested.

In view of the above remarks, Applicants respectfully submit that the present claims define subject matter that is patentable. The present claims also fully satisfy the requirements of 35 USC § 112, first and second paragraphs. Accordingly, the Examiner is respectfully requested to withdraw all rejections and allow the currently pending claims.

If the Examiner has any questions or comments, please contact Craig A. McRobbie, Reg. No. 42, 874 at the offices of Birch, Stewart, Kolasch & Birch, LLP at the number listed below.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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Attachments: Reference 1 and 2

Reference 1

IDENTIFICATION OF cDNA CLONES OF INTEREST

Methods of Screening

There are three methods to screen cDNA libraries for clones of interest:

- Nucleic acid hybridization
- Immunological detection of specific antigens
- Sib selection either by hybrid selection and translation of mRNA or by production of biologically active molecules

Most cloning projects today are aimed at isolating cDNAs corresponding to rare mRNAs and therefore require screening of large numbers of recombinant clones. This can be carried out effectively with only two types of reagents: antibodies and nucleic acid probes. In those rare instances when both types of reagents are available, nucleic acid probes are preferred because they can be used under a variety of different stringencies that minimize the chance of undesirable cross-reactions. Furthermore, nucleic acid probes will detect all clones that contain cDNA sequences, whereas antibodies will react only with a subset of these clones (in some cases one in six at best) in which the cDNA has been inserted into the vector in the correct reading frame and orientation. cDNA libraries that are to be screened by antibodies therefore need to be larger (by a factor of at least 6) than those that are to be screened by nucleic acid probes. Consequently, when using antibody probes to search for a cDNA clone corresponding to a mammalian mRNA present at the level of 1 molecule/cell or less, it is desirable to construct cDNA expression libraries that contain in excess of 10^7 members. This is not easy, especially when the amounts of mRNA are limited. Furthermore, screening a library of this size is expensive and laborious, and it becomes worthwhile to explore methods to enrich the mRNA (or cDNA derived from it) for the sequences of interest (see pages 8.6–8.10).

NUCLEIC ACID HYBRIDIZATION

This is the most commonly used and reliable method of screening cDNA libraries for clones of interest. None of the other methods displays such an abundance of attractive features. Screening by nucleic acid hybridization allows extremely large numbers of clones to be analyzed simultaneously and rapidly, does not require that the cDNA clones be full-length, and does not require that an antigenically or biologically active product be synthesized in the host cell. Furthermore, as a result of more than 20 years of work, the theoretical basis of nucleic acid hybridization is well-understood. This has led to the development of a large number of different techniques that can accommodate nucleic acid probes of very different lengths and specificities. Details of the methods for the preparation and use of these probes are presented in Chapters 10 and 11.

Homologous probes

Homologous probes contain at least part of the exact nucleic acid sequence of the desired cDNA clone. They are used in a variety of circumstances, for

example, when a partial clone of an existing cDNA is used to isolate a full-length clone from a cDNA library. Usually, a fragment derived from one end or the other of the existing clone is isolated, radiolabeled in vitro, and used to probe a library. Hybridization with homologous probes is always carried out under stringent conditions.

Partially homologous probes

Partially homologous probes are used to detect cDNA clones that are related, but not identical, to the probe sequences. If neither antibody nor nucleic acid probes are available, a number of alternative strategies can be considered. For example, if the same gene has already been cloned from another species or if a related gene has been cloned from the same species, it would be worthwhile carrying out a series of trial experiments to determine whether there is sufficient conservation of nucleic acid sequence to allow the screening of a cDNA library by hybridization. This is most easily accomplished by performing a series of Southern and northern hybridizations at different stringencies. For example, a large batch (50 μg) of genomic DNA is cleaved with a restriction enzyme that cleaves the probe sequence at one or two well-separated sites. It is a good idea to digest an equal amount of genomic DNA of the original species for use as a positive control. Aliquots (5–10 μg) of the digests are then applied to adjacent slots of a 0.8% agarose gel, electrophoresis is carried out, and the fragments are then transferred to a nitrocellulose filter as described in Chapter 9, pages 9.34–9.41. The filter is cut into strips, each of which is hybridized under different conditions to identical amounts of radioactive probe. For aqueous hybridization, the ionic strength of the solution is kept constant (usually 1 M Na^+) while the temperature of annealing is progressively lowered (from 68°C to 42°C). The strips are then washed extensively at the temperature of hybridization with a solution containing 2 × SSC, 0.5% SDS. When hybridization is carried out in solvents containing formamide, the temperature and ionic strength are usually kept constant (42°C and 6 × SSC [or 6 × SSPE], respectively) while the amount of formamide in the annealing buffer is progressively lowered from 50% to 0%. The strips are then washed extensively at 50°C in 6 × SSC, 0.5% SDS. A similar series of hybridizations can be carried out with mRNA preparations that have been fractionated by electrophoresis and transferred to a solid support. In both cases, the aim is to establish conditions that will allow the previously cloned gene to be used as a probe for the cDNA of interest, without undue interference from background hybridization.

Total cDNA probes

Total cDNA probes are prepared by uniform incorporation of radiolabeled nucleotides with reverse transcriptase or end-labeling of total or fractionated poly(A)$^+$ mRNA. They can be used to screen libraries of cDNA for specific clones *if* the cDNA clones of interest correspond to mRNA species present in the initial population at a frequency of at least 1 in 200 (see Gergen et al. 1979; Dworkin and Dawid 1980). It is not possible to detect cDNA clones homologous to species that are represented rarely in the mRNA preparation.

Reference 2

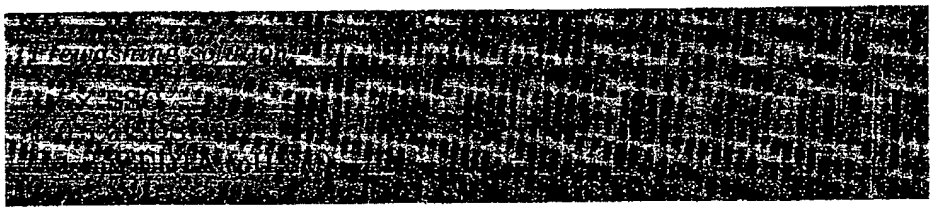
Hybridization to Nitrocellulose Filters Containing Replicas of Bacterial Colonies

The following protocol is designed for 30 circular nitrocellulose filters, 82 mm in diameter. Appropriate adjustments to the volumes should be made when carrying out hybridization reactions with different numbers or sizes of filters.

1. Float the baked filters on the surface of a tray of 2× SSC until they have become thoroughly wetted from beneath. Submerge the filters for 5 minutes.

2. Transfer the filters to a glass crystallizing dish containing at least 200 ml of prewashing solution. Stack the filters on top of one another in the solution. Cover the dish with Saran Wrap and transfer it to a rotating platform in an incubator. In this and all subsequent steps, the filters should be slowly agitated to prevent them from sticking to one another. Incubate the filters for 30 minutes at 50°C.

   Important: Do not allow the filters to dry at any stage during the prewashing, prehybridization, or hybridization steps.



3. Gently scrape the bacterial debris from the surfaces of the filters using Kimwipes soaked in prewashing solution. This reduces background hybridization without affecting the intensity or sharpness of positive signals.

4. Transfer the filters to 150 ml of prehybridization solution in a glass crystallizing dish. Incubate the filters for 1–2 hours at the appropriate temperature (i.e., 68°C when hybridization is to be carried out in aqueous solution; 42°C when hybridization is to be carried out in 50% formamide).

   Some workers prefer to incubate filters in heat-sealable plastic bags (Sears Seal-A-Meal or equivalent) (see, e.g., Chapter 9, page 9.53). This method avoids problems with evaporation and, because the sealed bags can be submerged in a water bath, ensures that the temperatures during hybridization and washing are correct. The bags must be opened and resealed when changing buffers. To avoid radioactive contamination of the water bath, the resealed bag containing radioactivity should be sealed inside a second, noncontaminated bag.

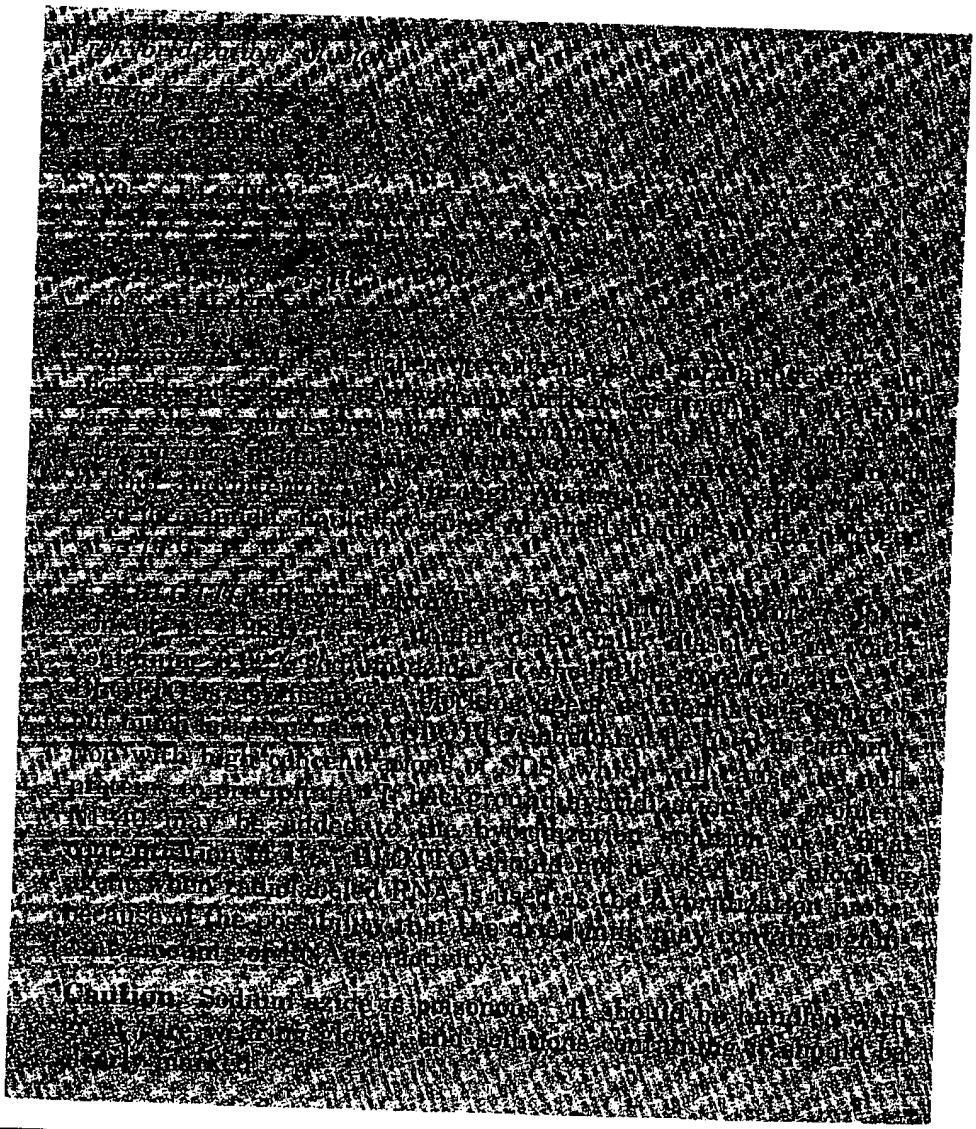
   Often, small bubbles of air form on the surface of the filter as the temperature of the prehybridization solution increases. It is important that these bubbles be removed by occasionally agitating the fluid in the bag; otherwise, the components of the prehybridization solution will not be able to coat the filter evenly. This problem can be minimized by heating the prehybridization solution to the appropriate temperature before adding it to the bag.

   The filters should be completely covered by the prehybridization solution. During prehybridization, sites on the nitrocellulose filter that nonspecifically bind single- or double-stranded DNA become blocked by proteins in the BLOTTO.

When ^{32}P-labeled cDNA or RNA is used as a probe, poly(A) at a concentration of 1 μg/ml should be included in the prehybridization and hybridization solutions to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

Whether or not to use a prehybridization solution containing formamide is largely a matter of personal preference. Both versions of these solutions give excellent results and neither has clear-cut advantages over the other. However, hybridization in 50% formamide at 42°C is less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. Offsetting this advantage is the two- to threefold slower rate of hybridization in solutions containing formamide.

To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6× SSC or 6× SSPE) at a temperature that is 20–25°C below T_m (see Chapter 9, pages 9.50–9.51). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6× SSPE is preferred because of its greater buffering power.



5. Denature ^{32}P-labeled double-stranded DNA probe by heating for 5 minutes to 100°C. Chill the probe rapidly in ice water. Single-stranded probe need not be denatured. Add the probe to the prehybridization solution covering the filters. Incubate at the appropriate temperature until $1–3 \times C_o t_{1/2}$ is achieved (see Chapter 9, page 9.48). During the hybridization, the containers holding the filters should be tightly closed to prevent the loss of fluid by evaporation.

   Alternatively, the probe may be denatured by adding 0.1 volume of 3 N NaOH. After 5 minutes at room temperature, transfer the probe to ice water and add 0.05 volume of 1 M Tris · Cl (pH 7.2) and 0.1 volume of 3 N HCl. Store the probe in ice water until it is needed.

   Between 2×10^5 and 1×10^6 cpm of ^{32}P-labeled probe (sp. act. $\geq 5 \times 10^7$ cpm/μg) should be used per milliliter of prehybridization solution. Using more probe will cause the background of nonspecific hybridization to increase; using less will reduce the rate of hybridization.

6. When the hybridization is completed, remove the hybridization solution and immediately immerse the filters in a large volume (300–500 ml) of 2× SSC and 0.1% SDS at room temperature. Agitate the filters gently, and turn them over at least once during washing. After 5 minutes, transfer the filters to a fresh batch of wash solution and continue to agitate them gently. Repeat the washing procedure twice more. At no stage during the washing procedure should the filters be allowed to dry.

   Hybridization mixtures containing radiolabeled single-stranded probes may be stored at 4°C for several days and reused without further treatment. However, hybridization mixtures containing complementary strands of DNA should be discarded since there is no satisfactory way to denature the double-stranded DNA that forms during the first round of hybridization.

7. Wash the filters twice for 1–1.5 hours in 300–500 ml of a solution of 1× SSC and 0.1% SDS at 68°C. At this point, the background is usually low enough to put the filters on film. If the background is still high or if the experiment demands washing at high stringencies, immerse the filters for 60 minutes in 300–500 ml of a solution of 0.2× SSC and 0.1% SDS at 68°C.

8. Dry the filters in the air at room temperature on paper towels. Arrange the filters (numbered side up) on a sheet of Saran Wrap. Apply adhesive dot labels marked with radioactive ink to several asymmetric locations on the Saran Wrap. These markers serve to align the autoradiograph with the filters. Cover the labels with Scotch Tape. This prevents contamination of the film holder or intensifying screen with the radioactive ink.

   Radioactive ink is made by mixing a small amount of ^{32}P with waterproof black drawing ink. We find it convenient to make the ink in three grades: very hot (>2000 cps on a hand-held minimonitor), hot (>500 cps on a hand-held minimonitor), and cool (>50 cps on a hand-held minimonitor). Use a fiber-tip pen to apply ink of the desired hotness to the adhesive labels. Attach radioactive-warning tape to the pen, and store it in an appropriate place.

9. Cover the filters with a second sheet of Saran Wrap. Expose the filters to X-ray film (Kodak XAR-2 or equivalent) for 12–16 hours at −70°C with an intensifying screen (see Appendix E).

10. Develop the film and align it with the filters using the marks left by the radioactive ink. Use a nonradioactive fiber-tip pen to mark the film with the positions of the asymmetrically located dots on the numbered filters. Tape a piece of clear Mylar or other firm transparent sheet to the film. Mark on the clear sheet the positions of positive hybridization signals. Also mark (in a different color) the positions of the asymmetrically located dots. Remove the clear sheet from the film. Identify the positive colonies by aligning the dots on the clear sheet with those on the agar plate.

    Some batches of nitrocellulose filters swell and distort during hybridization and subsequent drying, so that it becomes difficult to align the two sets of dots. This problem can be alleviated to some extent by autoclaving the dry filters between pieces of damp Whatman 3MM paper before use (10 lb/sq. in. for 10 minutes on liquid cycle). Nylon membranes do not suffer from this problem.

11. Using a sterile toothpick, transfer each positive bacterial colony into 1–2 ml of LB medium containing the appropriate antibiotic. Often, the alignment of the filters with the plate does not permit identification of an individual hybridizing colony. In this case, several adjacent colonies should be pooled. The culture is grown for several hours and then diluted and replated on agar plates so as to obtain approximately 500 colonies per plate. These colonies are then screened a second time by hybridization. A single, well-isolated, positive colony should be picked from the secondary screen and used for further analysis.

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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